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Apoptotic Vesicles Crossprime CD8 T Cells and Protect against Tuberculosis

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Summary

CD8 T lymphocytes are important effectors in protective immunity against *Mycobacterium tuberculosis*. We recently characterized the detour pathway of CD8 T cell activation in tuberculosis mediated by apoptotic vesicles from infected cells that transport mycobacterial antigens to dendritic cells (DCs). Here we demonstrate that apoptotic vesicles from mycobacteria-infected macrophages stimulate CD8 T cells in vivo. Homing of DCs to draining lymph nodes was critically required for effective crosspriming. Subsequent fate of vesicle-associated antigens in recipient DCs was characterized by endosomal mechanisms predominating over proteasomal processing. In addition, vesicle processing depended on the presence of saposins to disintegrate apoptotic membranes. Apoptotic vesicles displayed potent adjuvant activity by stimulating through Toll-like receptors (TLR). Ultimately, vaccination with vesicles from infected cells induced protection against *M. tuberculosis* infection. Taken together, we propose the detour pathway to represent a genuine immunological mechanism mediating crosspriming of CD8 T cells in vivo and protection against tuberculosis.

Introduction

Tuberculosis is the most prevalent bacterial infectious disease worldwide (Kaufmann and McMichael, 2005). Immunity against its etiologic agent, *Mycobacterium tuberculosis*, mainly involves CD4 T cells. Yet, CD8 T cells play a vital role in protection against tuberculosis, since lack of CD8 T cells causes higher susceptibility to mycobacterial challenge (Kaufmann, 2001). *M. tuberculosis* primarily infects macrophages of the lung. After infection, mycobacteria reside in early phagosomes and escape the immune system by inhibiting phagosome maturation and fusion with lysosomes (Russell, 2003). Moreover, mycobacterial antigens are segregated in phagosomes and do not traverse into the cytoplasm (Schaible et al., 2003). Since CD8 T cells recognize antigens originating from the cytosol to be processed along the MHC-I antigen-presentation pathway, the critical question has been how CD8 T cells encounter their respective mycobacterial epitopes. Previous work described the detour pathway of CD8 T cell activation in tuberculosis to overcome this obstacle (Schaible et al., 2003). Thus, mycobacteria induce apoptosis of infected macrophages, which subsequently release apoptotic vesicles containing mycobacterial antigens. These vesicles are engulfed by DCs, which process the antigenic cargo for subsequent presentation to CD8 T cells. Therefore, the detour pathway represents an exclusive mechanism of CD8 T cell activation by phagosome-enclosed antigens (Winau et al., 2004a).

Initially, the relation between apoptosis and T cell activation has been described in viral infection where dying infected cells promote priming of CD8 T cells by cocultured DCs (Albert et al., 1998). This mechanism involving transfer from a donor cell harboring the antigen to a recipient cell, eventually mediating antigen presentation, is referred to as crosspresentation or crosspriming related to T cell activation (Bevan, 1976). The detour pathway comprehends classical crosspriming, the latter being demonstrated for multiple tumor and infection models (Ackerman and Cresswell, 2004). Several pathogens including salmonellae and shigellae induce apoptosis of infected host cells (Sansonetti et al., 2000; Weinrauch and Zychlinsky, 1999). However, apoptotic vesicles as crosspriming agents have not been identified in these model systems. Different modes can explain the mechanism of crosspriming (Winau et al., 2005). Recent data demonstrated that the phagosome of antigen-presenting cells (APCs) is equipped with the complete molecular apparatus required for MHC-I-restricted antigen presentation including transporters associated with antigen presentation (TAP), the peptide loading complex, and adjacent proteasomes (Guermonprez et al., 2003; Houde et al., 2003). This suggests that the phagosome constitutes an autonomous organelle facilitating direct crosspresentation by the primary cell. However, novel studies could not confirm these findings (Touret et al., 2005; Groothuis and Neefjes, 2005).

Because the detour pathway was discovered in the human in vitro system, crosspriming of CD8 T cells could merely represent bystander activation by neighboring DCs taking up apoptotic vesicles in terms of an inflammatory process. To investigate whether the apoptotic vesicle path reflects a veritable immunological mechanism spanning uptake of vesicles by DCs in peripheral tissue, subsequent migration to draining lymph nodes, and priming of T cells in lymphatic tissue, we set up an

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antigen-specific in vivo model. To this end, we used recombinant *Mycobacterium bovis* BCG expressing the defined surrogate antigen ovalbumin (BCG-OVA) for infection of macrophages. Since inhibition of intrinsic infection-induced apoptosis of the host is not feasible, we regarded extrinsic control of vesicle formation upon cell death by infected APCs as the most reliable approach. We immunized mice with apoptotic vesicles from infected macrophages and adoptively transferred TCR-transgenic CD8 T cells specific for OVA to recipients, subsequently analyzing in vivo activation of CD8 T cells.

Results

Mycobacteria Induce Apoptotic Vesicles

After infection of macrophages, mycobacteria elicit regulated cell death of host cells (Schaible et al., 2003). In order to visualize apoptosis, we performed Annexin V staining of murine macrophages after infection with recombinant BCG-OVA. Annexin V binds to phosphatidylserine characteristically exposed on the outer leaflet of apoptotic membranes. Figure 1A reveals red fluorescence of Annexin V staining of BCG-OVA-infected macrophages, indicating apoptosis in contrast to noninfected cells. As control of apoptotic cell death, staurosporine was included, which activates caspase-3 and the subsequent apoptotic cascade.

Scanning electron microscopy (SEM) revealed vesicle formation of macrophages undergoing infection-induced apoptosis (Figure 1B). Remarkably, dying macrophages exhibited a string phenotype with apoptotic vesicles budding from cellular cords (Figure 1B, middle). Differential ultracentrifugation of apoptotic macrophage supernatants resulted in a homogenous vesicle preparation as indicated by SEM (Figure 1B, bottom).

To examine whether the surrogate antigen of recombinant BCG is present in apoptotic vesicles, we conducted immunoblots of vesicle protein with a monoclonal antibody against ovalbumin. Bands of ~12 kDa corresponded to the OVA fragment (amino acids 230–359) expressed by BCG and were absent in apoptotic vesicles from noninfected macrophages (Figure 1C). Taken together, the generation of apoptotic vesicles containing surrogate antigen from BCG-OVA-infected macrophages proved to be fully functional.

Crosspriming of CD8 T Cells by Apoptotic Vesicles

In order to investigate whether apoptotic vesicles activate naive CD8 T cells in vivo, we immunized wild-type mice subcutaneously with vesicle preparations from infected or naive macrophages. In parallel, we isolated CD8 T cells from TCR-transgenic animals (OT-1) specific for the OVA determinant SIINFEKL. Subsequently, we labeled the OT-1 cells with carboxyfluorescein diacetate succinimidylester (CFSE) before adoptive transfer to recipient immunized mice. This dye is equally partitioned upon cell division, allowing the generation of proliferation profiles. Three days after transfer, lymphocytes were prepared from draining lymph nodes and stained for CD8. Flow cytometric analysis revealed substantial activation of transferred CD8 T cells stimulated with vesicles from infected cells indicated by CFSE dilution (Figure 2A, top right). In contrast, CD8 T cells of recipient animals immunized with apoptotic vesicles from noninfected macrophages or vesicles from cells infected with wild-type BCG (not expressing OVA) did not proliferate (Figure 2A, top left, and data not shown). Moreover, intracellular cytokine staining demonstrated that CD8 T cells produced increasing amounts of interferon-γ (IFN-γ) upon division compared to OT-1 cells after stimulation with vesicles from noninfected cells (Figure 2A, bottom).

Next, we examined the impact of antigen-presenting molecules associated with apoptotic vesicles with regard to T cell activation. To this end, we generated vesicles lacking MHC-I molecules by infecting macrophages deficient in β_2 microglobulin (β_2m). Immunization with apoptotic vesicles from infected β_2m-KO macrophages induced proper CD8 T cell activation in vivo in contrast to stimulation with vesicles derived from uninfected cells (Figure 2B).

The use of apoptotic vesicles generated from infected wild-type cells for stimulation of transferred OT-1 cells in β_2m-deficient animals elicited a moderate CD8 T cell response (Figure 2C, top). However, the extent of CD8 T cell proliferation observed indicated potential transmission of MHC-I molecules from apoptotic vesicles to β_2m-deficient recipient cells lacking MHC-I proteins. To verify this notion, we conducted an in vitro assay employing β_2m-deficient DCs as APCs and $CD8^+$-sorted OT-1 lymphocytes as responder cells. When DCs lacking MHC-I were pulsed with apoptotic vesicles from BCG-infected macrophages, no antigen-specific activation of CD8 T cells was detectable. In marked contrast, addition of vesicles from BCG-OVA-infected cells induced robust T cell proliferation as determined by ^{3}H-thymidine incorporation. Stimulation of OT-1 cells with vesicles from BCG-OVA-infected macrophages in the absence of β_2m-deficient DCs failed to induce CD8 T cell activation (Figure 2C, bottom). Thus, a direct impact of apoptotic vesicles on OT-1 cells or OT-1 cell-mediated antigen presentation can be excluded.

Further, we analyzed the priming capacity of apoptotic vesicles with regard to other T cell populations. We performed adoptive transfer of TCR-transgenic CD4 T cells specific for OVA peptides in the context of MHC-II and immunized recipient mice with vesicles from infected or noninfected macrophages. Figure 2D demonstrates that apoptotic vesicles from infected cells potently activate CD4 T cells.

In conclusion, apoptotic vesicles efficiently crossprime CD8 T cells in vivo. In addition to antigen delivery, vesicles are able to transmit antigen-presenting molecules to recipient APCs. Vesicle-induced T cell activation includes priming of CD4 T cells.

The Detour Pathway Requires Homing of DCs

Because we observed CD8 T cell activation in draining lymph nodes after subcutaneous immunization with apoptotic vesicles, we aimed at elucidating the mode of transport of vesicles between peripheral and lymphatic tissue. Taking into account the two possibilities of vesicle transport either directly by the lymph or by a vehicle cell, we labeled apoptotic vesicles with a fluorochrome prior to subcutaneous injection in order to determine their destination. One day after application of labeled vesicles, we excised draining lymph nodes for cryosection. After staining the tissue with antibodies against the

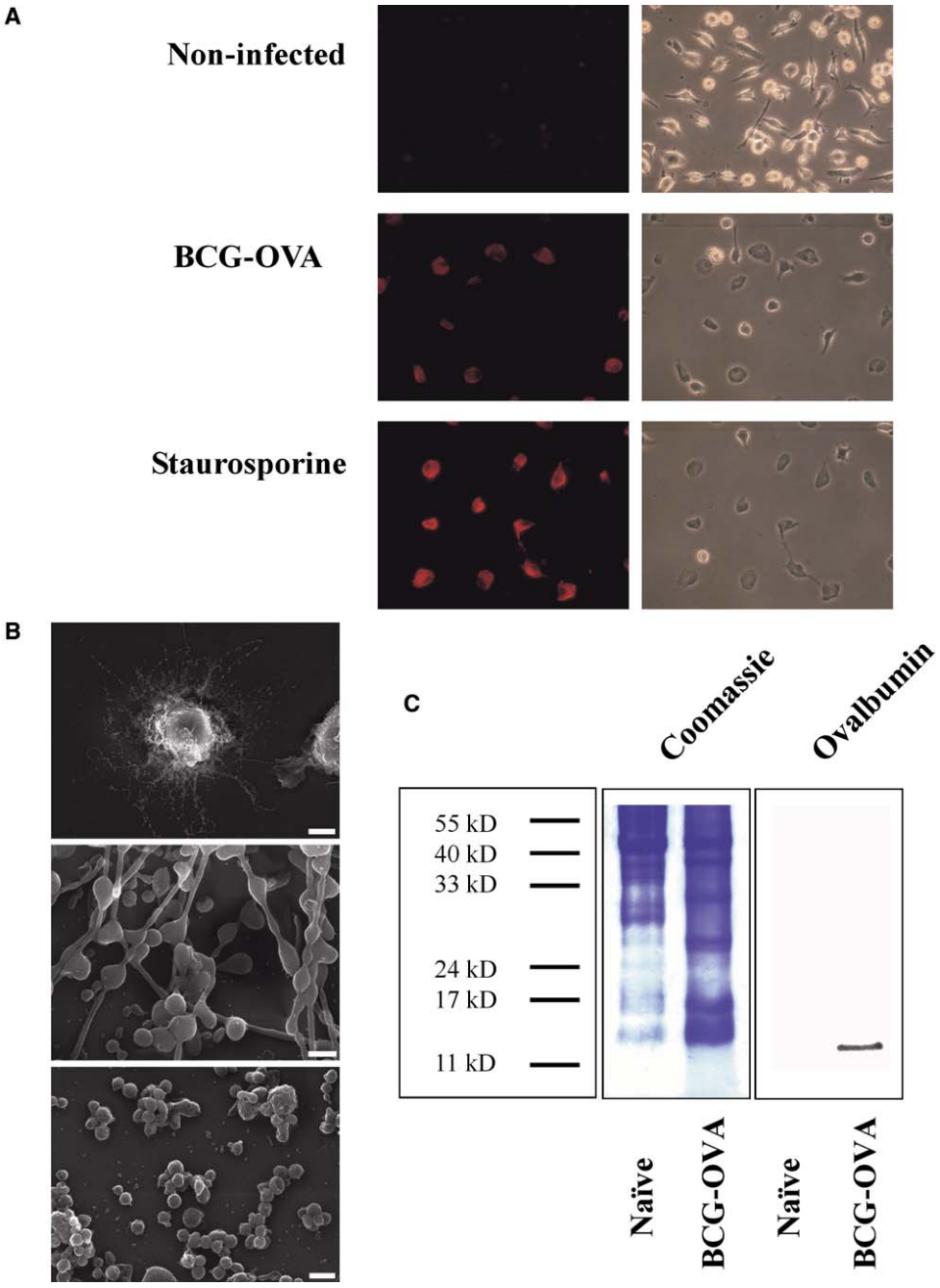

Figure 1. Mycobacteria Induce Apoptotic Vesicles

(A) Recombinant BCG elicits apoptosis of infected macrophages. Bone marrow-derived macrophages were infected with BCG-OVA at an moi of 10:1 for 4 hr. One day later, cells were fixed and stained with Annexin V (red fluorescence). As controls, macrophages were left uninfected or were treated with staurosporine. Left panels represent images by fluorescence microscopy and right panels are phase contrast.

(B) Dying macrophages exhibit a string phenotype. Macrophages were infected with BCG, fixed, and analyzed by scanning electron microscopy. The lower panel shows a preparation of apoptotic vesicles after differential ultracentrifugation of cell-culture supernatants from infected macrophages. Scale bars equal 5 μm in the upper panel, 500 nm in the lower panels.

(C) Vesicles from BCG-OVA-infected cells contain surrogate antigen. Apoptotic vesicle proteins were separated by SDS-PAGE, blotted to nitrocellulose, and stained with an ovalbumin antibody. A band of ~12 kDa corresponding to the OVA fragment is demonstrated for apoptotic vesicles from infected macrophages in contrast to naive vesicles. Coomassie-stained bands represent the corresponding protein gel.

DC marker CD11c and the macrophage-specific surface protein F4/80, we examined the lymph node sections under a confocal microscope. Figure 3A shows the mutually exclusive staining of DCs ($CD11c^+$) and macrophages ($F4/80^+$) in lymph node sections (top left). Lines of red fluorescent vesicles were detected in draining lymph nodes after subcutaneous injection (Figure 3A, bottom middle). The location of labeled apoptotic vesicles superimposed on the staining for DCs (Figure 3A, bottom left). The overlay of DCs and vesicles largely

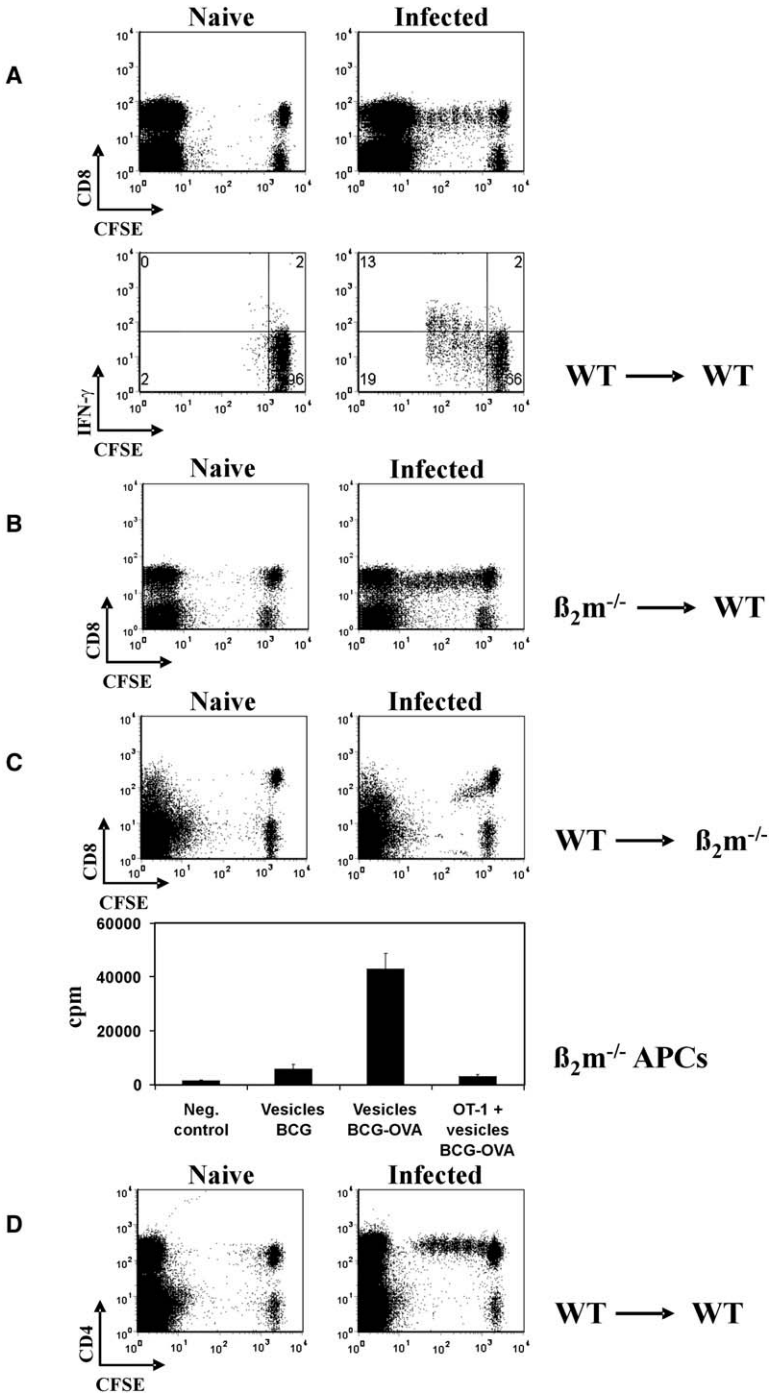

Figure 2. Crosspriming of T Cells by Apoptotic Vesicles

(A) CD8 T cell activation by apoptotic vesicles in vivo. Wild-type mice were immunized with apoptotic vesicles from infected (Infected) or noninfected (Naive) macrophages and received in parallel CFSE-labeled OT-1 cells by adoptive transfer. Three days after stimulation, cells were isolated from draining lymph nodes and stained for CD8 and intracellular IFN-γ. Left dot plots represent stimulation with naive vesicles (Naive), right dot plots show activation by vesicles from infected cells (Infected). Lower panels are gated on $CD8^+$ T cells. wt → wt = vesicles from wild-type donor macrophages injected into wild-type recipient mice. Mean of 45% (±5%; Infected) or 4% (±2%; Naive) dividing cells of total $CFSE^+$ CD8 T cells from three independent experiments with similar results.

(B) MHC-I molecules on apoptotic vesicles are not required for CD8 T cell activation. After a similar experimental set-up as described in (A), we immunized mice with apoptotic vesicles from β_2m-deficient macrophages. Left dot plot represents stimulation with naive (Naive), right dot plot with vesicles from infected (Infected) cells. $\beta_2m^{-/-}$ → wt = vesicles from $\beta_2m^{-/-}$ donor macrophages injected into wild-type recipient mice. Mean of 54% (±9%; Infected) or 8% (±4%; Naive) dividing cells of total $CFSE^+$ CD8 T cells from three independent experiments with similar results.

(C) Apoptotic vesicles transmit MHC-I molecules to recipient APCs. By a similar approach as in (A) and (B), we immunized β_2m-deficient mice with apoptotic vesicles from wild-type macrophages. Left dot plot: stimulation with naive vesicles (Naive); right dot plot: activation with vesicles from infected cells (Infected). wt → $\beta_2m^{-/-}$ = vesicles from wild-type donor macrophages injected into $\beta_2m^{-/-}$ recipient mice. The lower graph shows an in vitro experiment with β_2m-deficient DCs as APCs in coculture with OT-1 cells. Irradiated DCs were left untreated (Neg. control) or were pulsed with vesicles from BCG- or BCG-OVA-infected macrophages. Additionally, OT-1 cells only were stimulated with vesicles from BCG-OVA-infected cells. Two days after incubation, antigen-specific proliferation was determined by ^{3}H-thymidine incorporation as depicted by counts per minute (cpm).

(D) Activation of CD4 T cells by apoptotic vesicles. Three days after immunization of mice with vesicles and adoptive transfer of CFSE-labeled OT-2 cells, lymph node cells were isolated and stained for CD4. Left dot plot represents stimulation with naive vesicles (Naive), right dot plot activation with vesicles from infected cells (Infected). wt → wt = vesicles from wild-type donor macrophages injected into wild-type recipient mice. Mean of 50% (±10%; Infected) or 5% (±4%; Naive) dividing cells of total $CFSE^+$ CD4 T cells from three independent experiments with similar results.

demonstrated colocalization, indicating that apoptotic vesicles were taken up by DCs (Figure 3A, bottom right). Remarkably, resident lymph node cells such as macrophages did not contain labeled material (Figure 3A, top right), suggesting that DCs engulfed vesicles in the periphery and subsequently migrated to the draining lymph node.

In addition to these morphological data, we performed functional experiments on homing of DCs. We took advantage of mice deficient for chemokine receptor 7 (CCR7), which is expressed by naive and memory T cells as well as mature DCs as prerequisite for homing to lymphatic tissue (Martin-Fontecha et al., 2003). We immunized CCR7-deficient animals with apoptotic

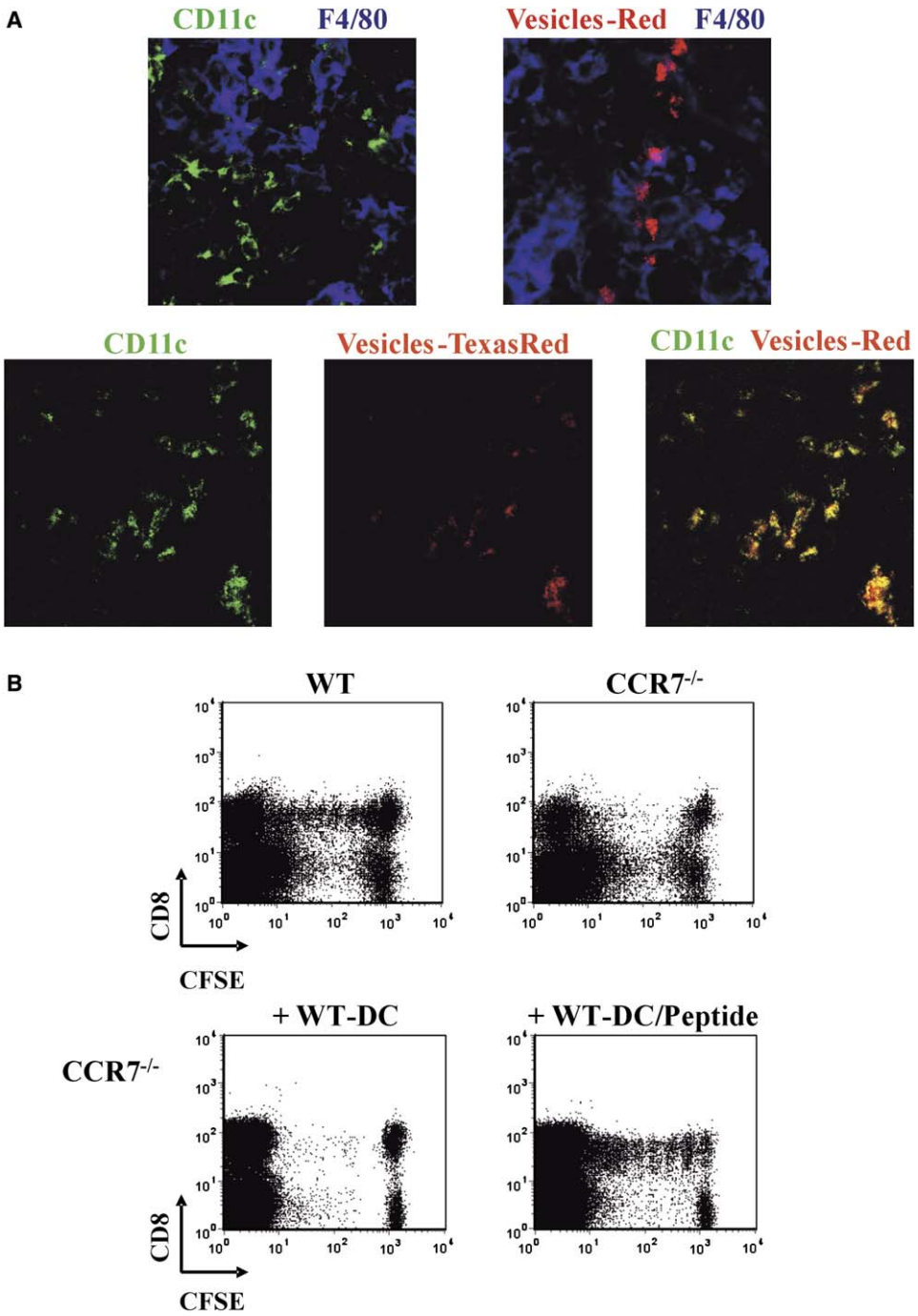

Figure 3. The Detour Pathway Requires Homing of DCs

(A) Apoptotic vesicles are allocated to DCs. The upper left confocal picture shows an overlay of a lymph node section stained for the DC marker CD11c (green) and the macrophage-specific molecule F4/80 (blue). The upper right picture represents an overlay demonstrating the presence of red fluorescent material in the draining lymph node after subcutaneous injection of labeled apoptotic vesicles and staining for the macrophage marker F4/80 (blue). The lower left confocal picture shows a lymph node section stained for CD11c (green). The lower middle image demonstrates red fluorescent apoptotic vesicles in the same draining lymph node section. Both images are merged to overlay, depicted in the lower right panel. (B) Homing of DCs is required for crosspriming of CD8 T cells. Wild-type (wt) and CCR7-deficient ($CCR7^{-/-}$) mice were subcutaneously immunized with apoptotic vesicles from mycobacteria-infected macrophages and received CFSE-labeled OT-1 cells by adoptive transfer (top). As controls, CCR7-deficient animals ($CCR7^{-/-}$) received CFSE-labeled OT-1 cells and wild-type DCs (+ WT-DC) unpulsed or pulsed with SIINFEKL peptide (+ WT-DC/Peptide; bottom). Three days after stimulation, cells from draining lymph nodes were isolated and stained for CD8.

vesicles from BCG-OVA-infected macrophages and adoptively transferred CFSE-labeled OT-1 cells in parallel. Three days after transfer, cells from lymph nodes were isolated and stained for CD8. Analysis by flow cytometry revealed lack of CD8 T cell activation in CCR7-knockout mice in contrast to the proliferative response

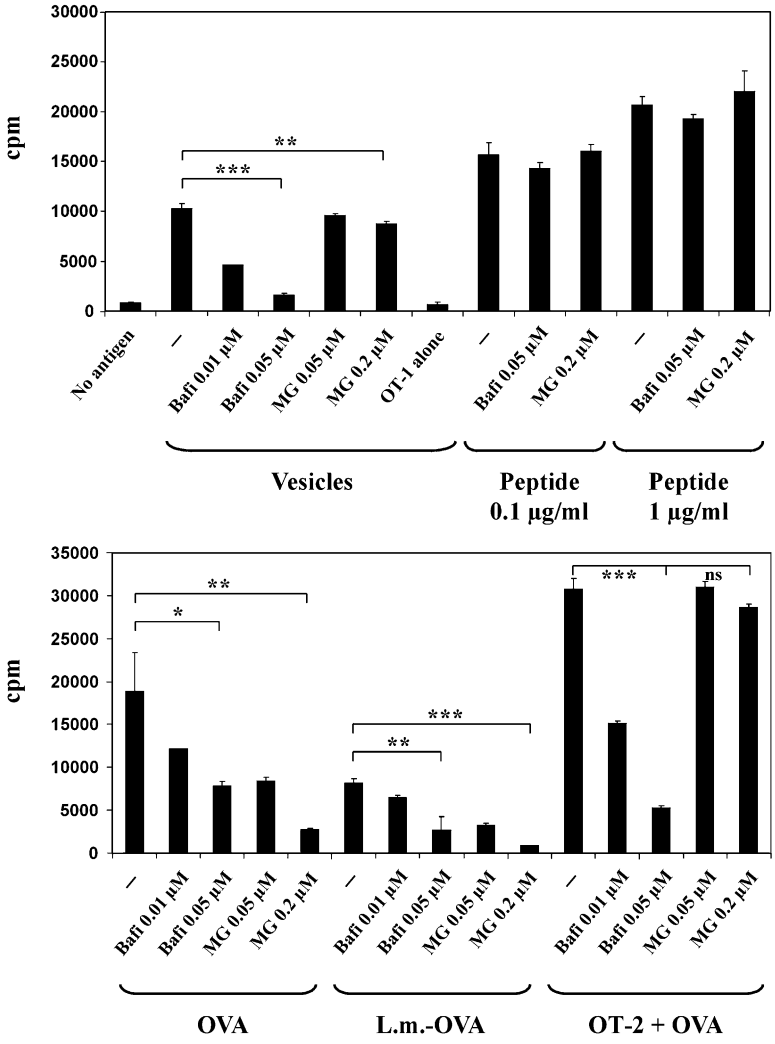

Figure 4. Endosomal Predominates over Proteasomal Processing of Apoptotic Vesicles

Irradiated DCs were pulsed with vesicles from BCG-OVA-infected macrophages (Vesicles) or left untreated (No antigen) before coculture with $CD8^+$-sorted OT-1 cells in vitro. Moreover, APCs were incubated with bafilomycin (Bafi) or MG 132 (MG) in low or high concentrations before addition of T cells. OT-1 cells only (OT-1 alone) incubated with apoptotic vesicles from BCG-OVA-infected macrophages were also included. In addition, DCs were pulsed with low or high peptide concentrations or soluble ovalbumin (OVA; 5 µg/ml), or were infected with L.m.-OVA at a moi of 5 for 30 min before incubation with inhibitors and CD8 or CD4 T cells (OT-2). Two days after stimulation, antigen-specific CD8 or CD4 T cell proliferation was measured by ^{3}H-thymidine incorporation as depicted by counts per minute (cpm). Data are representative for two independent experiments with similar results. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as analyzed by Student's t test.

in wild-type animals (Figure 3B, top). To exclude an intrinsic deficiency of $CCR7^{-/-}$ mice to prime wild-type CD8 T cells, we transferred wild-type DCs unpulsed or pulsed with SIINFEKL peptide in parallel to OT-1 cells to $CCR7^{-/-}$ recipients. Three days after adoptive transfer, flow cytometric analysis revealed proper antigen-specific CD8 T cell activation (Figure 3B, bottom). Taken together, our functional experiments in the CCR7-deficient system verify the morphological data and indicate that homing of DCs is critically required for crosspriming of CD8 T cells by apoptotic vesicles.

Processing of Apoptotic Vesicles in DCs

Since DCs emerged as central to crosspriming of CD8 T cells by apoptotic vesicles, we further focused on differential processing of vesicles in APCs. An in vitro T cell assay was set up with bone marrow-derived DCs as APCs in coculture with $CD8^+$-purified OT-1 cells as responder T cells. After pulsing DCs with apoptotic vesicles derived from BCG-OVA-infected macrophages, CD8 T cells responded with antigen-specific proliferation as measured by ^{3}H-thymidine incorporation of dividing OT-1 cells. Treatment of DCs with bafilomycin prior to incubation with vesicles and addition of T cells abrogated the CD8 T cell response in a dose-dependent manner (Figure 4, top). Bafilomycin blocks the endosomal H^+-ATPase, which facilitates acidification of late endosomes and lysosomes. Thus, inhibition of OT-1 cell stimulation by bafilomycin demonstrated the importance of endosomal processing of apoptotic vesicles for CD8 T cell activation. Preincubation of APCs with the proteasome inhibitor MG 132 had only a minor impact on T cell activation (Figure 4, top). In order to exclude effects on antigen presentation of cells present among CD8 T cells, we pulsed OT-1 cells with vesicles from infected macrophages that failed to induce T cell activation. To exclude toxic effects of the chemical inhibitors used, we pulsed DCs with low (0.1 µg/ml) or high (1 µg/ml) concentrations of SIINFEKL peptide prior to incubation with bafilomycin or MG 132 and subsequent addition of CD8 T cells. Since impairment of the T cell response to peptide presentation was not observed, any toxic effect of the inhibitors can be excluded (Figure 4, top). The use of ovalbumin (5 µg/ml) in the same experimental set-up revealed abrogation of the T cell response in the presence of MG 132, indicating the importance of proteasomes in crosspriming of CD8 T cells by soluble protein (Figure 4, bottom). To assess the efficacy of proteasome and lysosomal acidification block, we infected DCs with recombinant *Listeria*

monocytogenes expressing OVA (L.m.-OVA) at a moi of 5 in the presence of the inhibitors. Due to the action of listeriolysin, listeria are able to induce membrane pores escaping the phagosome to reach the cytosol rapidly after infection. This enabled us to study the impact of MG 132 on processing of cytosolic protein. Figure 4 (bottom) demonstrates the complete block of antigen recognition due to inhibition of the proteasome. Of note, neutralization of the endosomal compartment also reduced T cell activation since listeriolysin deploys optimal activity in an acidic milieu. Finally, to investigate the impact of bafilomycin and MG 132 on MHC-II restricted antigen presentation, we pulsed DCs with ovalbumin (5 μg/ml) prior to incubation with the inhibitors and addition of ovalbumin-specific CD4 T cells (OT-2). Inhibition of endosomal acidification efficiently abrogated antigen recognition by CD4 T cells, whereas proteasome block had no effect (Figure 4, bottom). Taken together, endosomal predominated over proteasomal mechanisms concerning processing of antigen contained in apoptotic vesicles for subsequent CD8 T cell induction.

Since we identified the lysosomal compartment as vital for antigen editing of apoptotic vesicles, we questioned whether other lysosomal helper molecules were required for vesicle processing. Previous studies described saposins as lipid transfer proteins located in the lysosome that interact with intralysosomal vesicles (Schuette et al., 2001; Kolter and Sandhoff, 2005). Thereby saposins destabilize membrane structures and expose their constituents for further processing (Vaccaro et al., 1995). Apoptotic and intralysosomal vesicles exhibit some analogy in structure. Moreover, saposins possess high affinity for phosphatidylserine classically exposed on the surface of apoptotic membranes. Thus, we proposed a role of saposins in disintegration of apoptotic vesicles and performed experiments with DCs from mice lacking prosaposin, the precursor protein of saposins A-D, for CD8 T cell activation. When APCs generated from prosaposin-deficient animals were pulsed with different concentrations of vesicles from BCG-OVA-infected macrophages, the CD8 T cell response was significantly reduced compared to stimulation with heterozygous or wild-type control DCs (Figure 5). After reconstitution of prosaposin-deficient APCs by addition of recombinant prosaposin to cell culture, the T cell response was largely restored. Loading of prosaposin-knockout APCs with different concentrations of SIINFEKL peptide induced a vigorous CD8 T cell response, which showed an otherwise physiological MHC-I-restricted antigen presentation capacity (Figure 5). Moreover, addition of soluble ovalbumin (5 μg/ml) revealed an equal magnitude of T cell activation by prosaposin-deficient APCs compared to heterozygous or wild-type DCs (Figure 5). Thus, a generalized defect in antigen processing due to absence of prosaposin can be excluded. Additionally, we previously demonstrated that the lysosomal compartment of prosaposin-deficient cells is anatomically intact and that apart from α-glucosidase function, they exhibit normal activity of lysosomal enzymes such as β-hexosaminidase and β-galactosidase (Winau et al., 2004b). Taken together, lack of prosaposin impaired CD8 T cell activation, demonstrating a role of saposins in processing of apoptotic vesicles.

Apoptotic Vesicles Are Adjuvants

Productive T cell responses depend on sufficient antigen presentation by potent APCs like macrophages and DCs (Mellman and Steinman, 2001; Shortman and Liu, 2002). Successful T cell priming is facilitated by activation of APCs. Pathogens possess conserved molecules such as lipopolysaccharide (LPS) or bacterial lipoprotein (BLP), which stimulate APCs through Toll-like receptors (TLR) (Medzhitov, 2001). We determined whether apoptotic vesicles from BCG-infected macrophages are equipped with mycobacterial ligands that stimulate APCs through TLRs. To this end, we used the stably transfected 293 HEK cell line expressing TLR-2, which was transiently cotransfected with an NF-κB-regulated luciferase reporter plasmid. After TLR-2 ligation, recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88) leads to NF-κB activation and subsequent expression of luciferase. One day after cotransfection, cells were incubated with increasing amounts of apoptotic vesicles from infected or noninfected macrophages. After 6 hr, cells were lysed and tested for NF-κB-dependent luciferase activity. Apoptotic vesicles from mycobacteria-infected macrophages elicited NF-κB activation in a dose-dependent manner in contrast to vesicles from uninfected cells (Figure 6A). In order to extend our insights from a reporter cell system to more natural conditions, we stimulated primary APCs deficient for TLR-2, TLR-4, or MyD88 with apoptotic vesicles. DCs or macrophages from the respective knockout animals were pulsed either with vesicles from infected or noninfected cells or BCG. Subsequently, cell cultures were analyzed for IL-12 or TNF-α secretion. Vesicles from infected cells induced significant amounts of IL-12 in wild-type DCs, which was largely reduced in TLR-2-, TLR-4-, and MyD88-KO DCs, whereas stimulation with BCG led to IL-12/IL-23 p40 secretion by wild-type as well as TLR-4-KO DCs (Figure 6B). Vesicles also failed to induce TLR-2-deficient macrophages to secrete TNF-α in contrast to wild-type or TLR-4-KO macrophages (Figure 6C). Consistent with this finding, cells lacking the adaptor molecule MyD88 were not activated by vesicles from infected macrophages. In conclusion, apoptotic vesicles from mycobacteria-infected cells exhibit strong adjuvanticity by activation of APCs through TLR-2 engagement.

In order to characterize the mycobacterial content of apoptotic vesicles, we performed immunoblots with protein from vesicles derived from mycobacteria-infected compared to noninfected cells. Figure 6D demonstrates abundant bands of mycobacterial proteins present in vesicles detected by a polyclonal anti-BCG serum. One of these proteins corresponded to a ~31 kDa band reflecting Ag85, a protective antigen shared by *M. tuberculosis* and BCG (Huygen et al., 1996), as revealed by a specific antibody (Figure 6D). Additionally, molecules were detected by immunoblot that represent both antigens and adjuvants. Thus, mycobacterial 19 kDa lipoprotein, which binds to TLR-2 and serves as T cell antigen (Lopez et al., 2003), was present in apoptotic vesicles from infected macrophages (Figure 6D). Taken together, mycobacterial proteins such as the 19 kDa lipoprotein are constituents of the vesicular protein content and confer strong adjuvanticity to apoptotic vesicles.

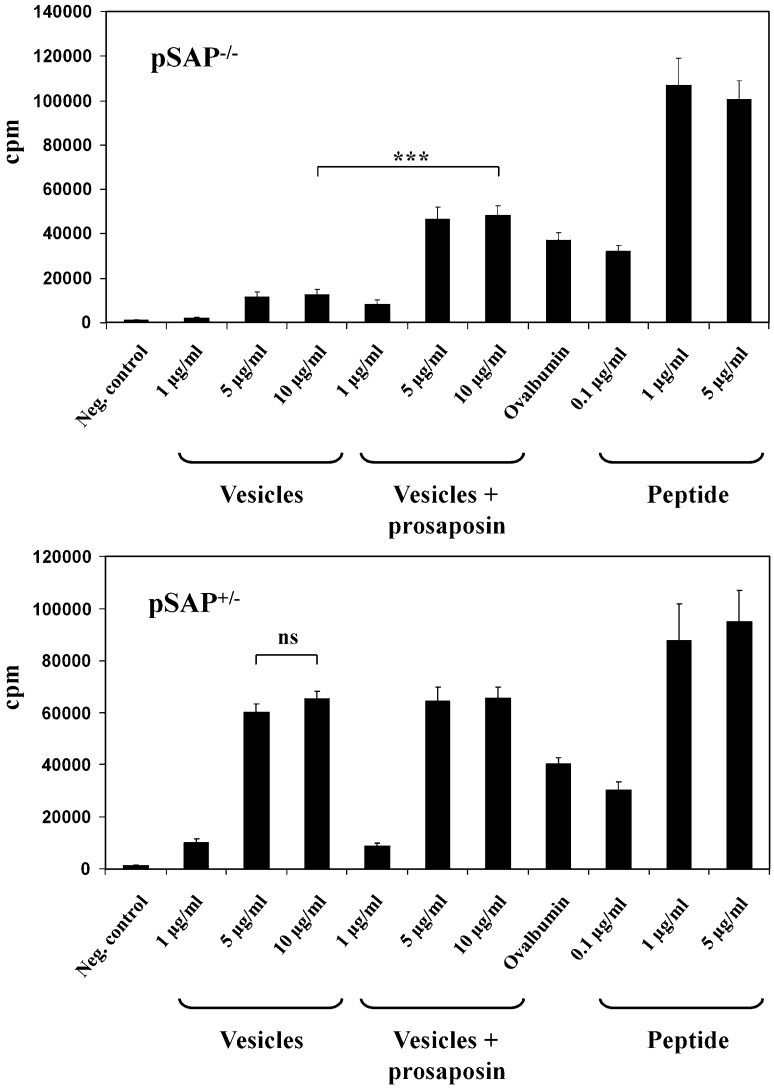

Figure 5. Saposins Play a Fundamental Role in Disintegration of Apoptotic Vesicles

Prosaposin-deficient ($pSAP^{-/-}$) DCs were pulsed with different concentrations of apoptotic vesicles from BCG-OVA-infected macrophages and incubated with OT-1 cells compared to DCs from heterozygous ($pSAP^{+/-}$) mice. Additionally, DCs were reconstituted with prosaposin 24 hr before the assay. Different concentrations of SIINFEKL were used to pulse DCs as antigen presentation controls. Soluble ovalbumin (5 µg/ml) was added to control normal function of antigen processing. Two days after stimulation, antigen-specific CD8 T cell proliferation was measured by ^{3}H-thymidine incorporation as depicted by counts per minute (cpm). Graphs are representative for three independent experiments with similar results. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as analyzed by Student's t test.

Vaccination with Apoptotic Vesicles Protects against Tuberculosis

Because apoptotic vesicles from mycobacteria-infected cells carry antigen, antigen-presenting molecules, and adjuvants, they are able to efficiently activate CD4 as well as CD8 T cells. Therefore, we examined the protective efficacy of the vesicle-based vaccine against infection with *M. tuberculosis*. We vaccinated C57BL/6 mice subcutaneously twice at an interval of 10 days with apoptotic vesicles from BCG-infected or noninfected macrophages. As positive controls, we included animals immunized with BCG, the current vaccine against tuberculosis. 10 days after the last immunization, we challenged mice with a low inoculum *M. tuberculosis* by aerosol infection. On day 20 and 50 postinfection, we collected lungs, livers, and spleens from experimental animals and plated homogenized organs in serial dilutions on 7H11 agar. Three weeks later, *M. tuberculosis* colonies were counted and CFU per ml organ were determined. Vesicles from noninfected cells failed to protect against *M. tuberculosis* infection comparable to the nonvaccinated group (Figure 7). In marked contrast, vaccination with apoptotic vesicles from mycobacteria-infected macrophages induced protection against aerosol challenge as determined in lung and spleen both at day 20 and day 50 in the order of BCG-induced protection (Figures 7A–7C). Differences in CFU between groups vaccinated with vesicles from infected macrophages or BCG and nonvaccinated groups or those vaccinated with vesicles from noninfected macrophages were statistically significant. Due to spontaneous reduction of bacterial burden of nonvaccinated mice, lung CFU at day 50 were higher in nonvaccinated compared to vaccinated animals while not statistically significant (Figure 7B). However, at the same time point after infection, protection induced by the vesicle-based vaccine was significant in spleen compared to the nonvaccinated group (Figure 7C). To conclude, vaccination with apoptotic vesicles protects against tuberculosis.

Discussion

The aim of this study was to investigate whether the detour pathway of CD8 T cell activation is operative in vivo. Since previous work focused on analysis of human in vitro systems (Schaible et al., 2003), the question

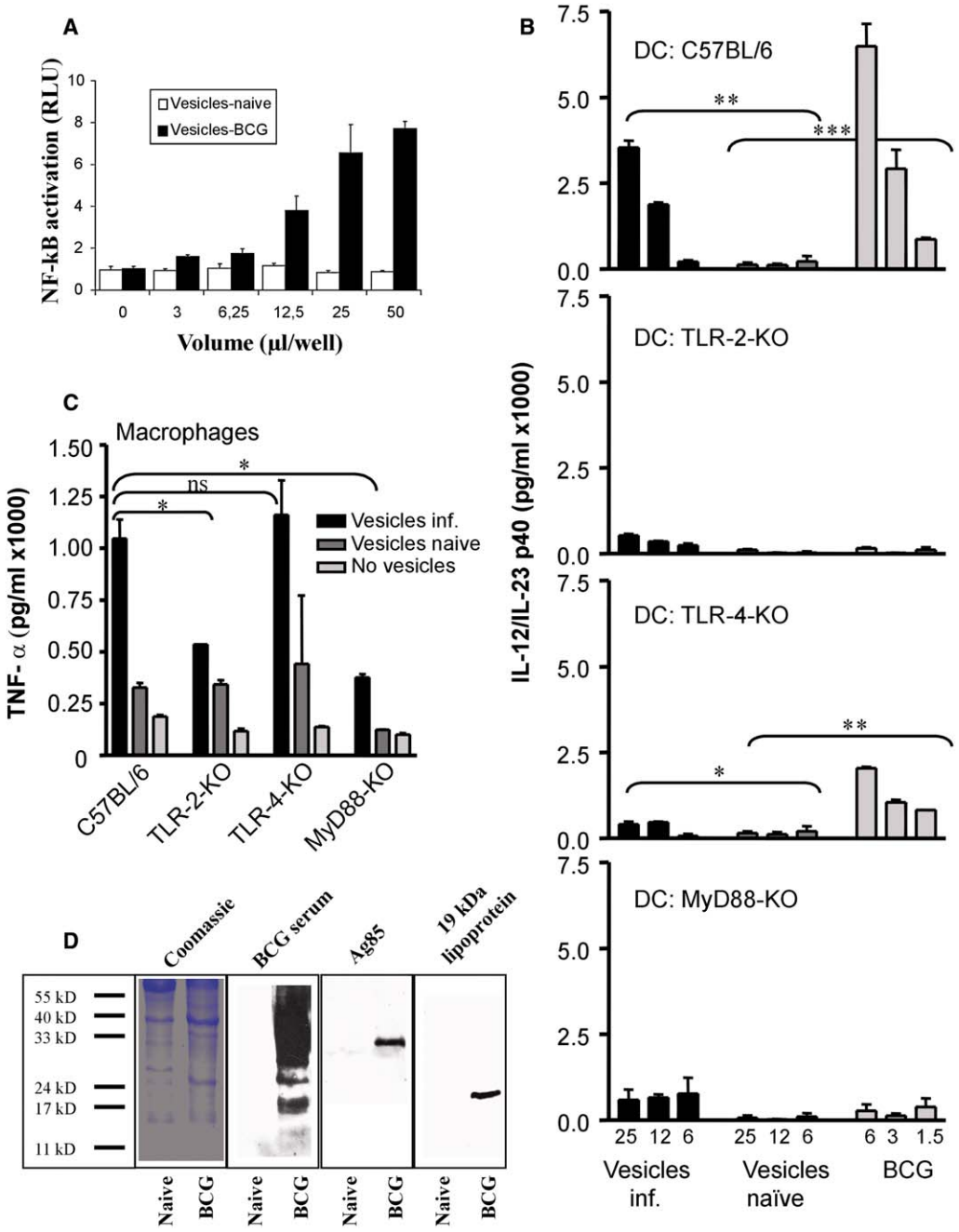

Figure 6. Apoptotic Vesicles Are Adjuvants

(A) TLR-2 binds apoptotic vesicles. 293 HEK cells constitutively expressing TLR-2 were transiently cotransfected with an NF-κB-regulated luciferase reporter plasmid. One day after transfection, cells were incubated with vesicles from mycobacteria-infected (Vesicles-BCG) or noninfected (Vesicles-naive) macrophages. 6 hr after stimulation, cells were lysed and luciferase activity was determined. Ordinate represents induction of NF-κB activation indicated by relative luciferase units (RLU).

(B and C) Apoptotic vesicles activate primary APCs through TLR-2. Bone marrow-derived DCs (B) and macrophages (C) generated from wild-type C57BL/6, TLR-2-, TLR-4-, or MyD88-knockout animals were incubated with either vesicles from infected or noninfected cells or BCG. Protein concentrations of all three preparations were adjusted to 5 mg/ml. After stimulation for 1 (macrophages) or 2 (DCs) days, supernatants were harvested for detection of IL-12/IL-23 p40 (B) or TNF-α (C) by ELISA. Ordinate indicates cytokine concentration in pg/ml. Data are representative for three independent experiments with similar results. *$p < 0.03$; **$p < 0.004$; ***$p < 0.0004$ as analyzed by Student's t test.

(D) Apoptotic vesicles contain mycobacterial antigens and adjuvants. Apoptotic vesicle protein from infected macrophages or naive cells was separated by SDS-PAGE, blotted to nitrocellulose, and stained with anti-BCG serum, Ag85, or 19 kDa lipoprotein antibodies. The size of the bands detected can be deduced from the corresponding protein standard. Coomassie-stained bands represent the corresponding protein gel.

remained whether the apoptotic vesicle path represents a unique immunological mechanism instead of mere bystander activation characteristic for inflammatory processes. Thus, we established an antigen-specific model with apoptotic vesicles from BCG-OVA-infected macrophages for immunization of mice receiving OVA-specific

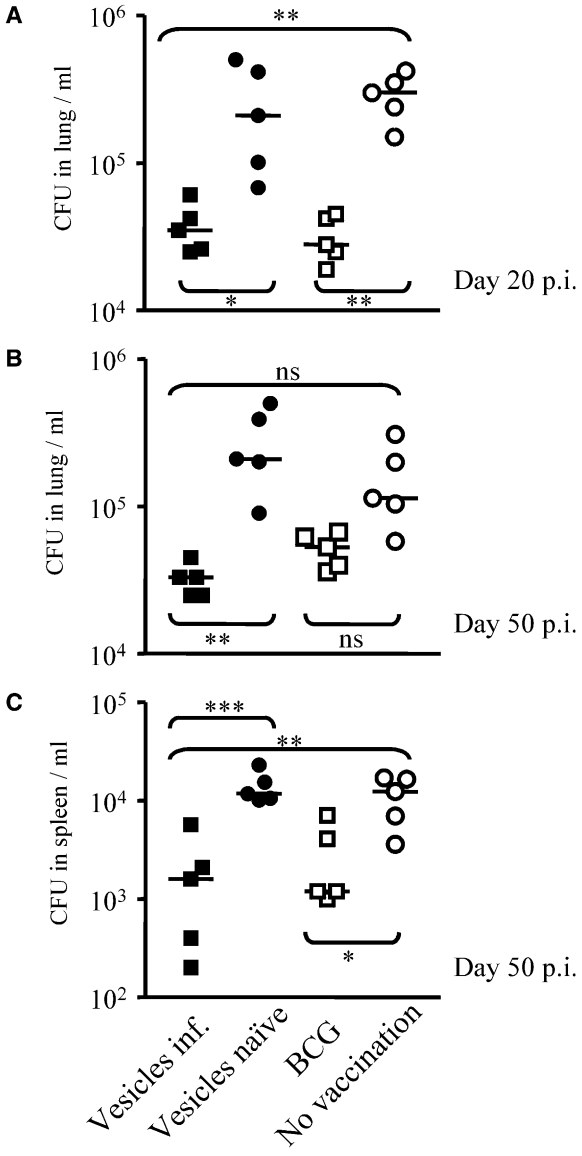

Figure 7. Vaccination with Apoptotic Vesicles Protects against Tuberculosis

Mice were vaccinated subcutaneously with apoptotic vesicles from infected or noninfected macrophages. As controls, animals were immunized with the standard vaccine BCG or were left untreated. 10 days after the last immunization, mice were challenged with *M. tuberculosis* aerosol infection (138 CFU per mouse). 20 and 50 days after challenge, lungs and spleens were collected, homogenized, and plated on agar in serial dilution. Three weeks later, *M. tuberculosis* colonies were counted.

(A) Ordinate represents *M. tuberculosis* CFU/ml in lungs at day 20 for the depicted experimental groups.

(B) Ordinate represents CFU/ml in lungs determined 50 days postinfection.

(C) Ordinate represents the CFU/ml in spleens at day 50 after infection. Colony counts in spleens at day 20 postinfection were low and heterogenous but revealed a similar trend (data not shown).

*p < 0.05; **p < 0.01; ***p < 0.001; ns, no significance, as analyzed by Tukey's multiple-comparison test.

CD8 T cells by adoptive transfer. The detour pathway involves induction of apoptosis by *M. tuberculosis* upon infection of macrophages, which subsequently release apoptotic vesicles containing mycobacterial antigens. DCs engulf these vesicles for further processing and activation of CD8 T cells by antigens otherwise segregated in phagosomes (Winau et al., 2004a). Our studies of infection-induced apoptosis by scanning electron microscopy revealed a string phenotype of mycobacteria-infected macrophages showing vesicles blebbing from cellular cords.

Subcutaneous immunization with apoptotic vesicles from mycobacteria-infected macrophages induced CD8 T cell activation in draining lymph nodes. Thus, crosspriming by apoptotic vesicles occurs in vivo. Despite conflicting data in some viral and tumor systems (Ochsenbein et al., 2001; Freigang et al., 2003; Zinkernagel, 2002), the importance of crosspriming has been established in numerous models with tumors, infections, and defined antigens like OVA (Thomas et al., 2004; Storni and Bachmann, 2004; Ackerman and Cresswell, 2004). Crosspriming initiated by pathogen-induced apoptosis has been described for infection with viruses and bacteria (Albert et al., 1998; Yrlid and Wick, 2000). Several pathogens induce apoptosis in host cells (Weinrauch and Zychlinsky, 1999), suggesting that the detour pathway represents a general mechanism for crosspriming of CD8 T cells in infectious disease. In *Listeria monocytogenes* infection, a crucial role of neutrophils in crosspriming of listeria-specific CD8 T cells has been shown (Tvinnereim et al., 2004). Moreover, neutrophils are primary target cells of listeriae and other pathogenic invaders and undergo apoptosis upon infection. Thus, it is likely that the detour pathway is valid for a multitude of crosspriming conditions. However, apoptotic vesicles have not been identified in these models. Different vesicle structures have been described as efficient agents in T cell crosspriming, termed exosomes (Thery et al., 2002). These vesicles represent secreted lysosomes, constitutively released by cells independently from cell death. Exosomal transfer of antigens as part of the detour pathway has been excluded, since inhibition of apoptosis in donor macrophages totally abrogates activation of CD8 T cells (Schaible et al., 2003).

CD8 T cells activated by apoptotic vesicles from mycobacteria-infected cells produced IFN-γ, which is central to control of tuberculosis (Kaufmann, 2001). Notably, IFN-γ stimulates uninfected macrophages to produce reactive nitrogen intermediates in order to effectively kill intracellular *M. tuberculosis*. Moreover, we examined the impact of antigen-presenting molecules associated with apoptotic vesicles on T cell activation. When mice were immunized with vesicles from β_2m-deficient macrophages devoid of MHC-I surface expression, proper CD8 T cell activation was observed as indicated by antigen-specific proliferation. Thus, the presence of MHC-I molecules on apoptotic vesicles is not required for crosspriming. Vesicles from wild-type macrophages induced definite, but weak, CD8 T cell proliferation in β_2m-KO-recipient mice, suggesting that transfer of MHC-I molecules from apoptotic vesicles to recipient APCs occurred in vivo. However, the weak T cell response could be due to quantitatively minor transfer of MHC-I proteins. In order to verify MHC-I transmittance, we pulsed DCs from β_2m-KO mice with apoptotic vesicles for subsequent T cell stimulation in vitro. Indeed, apoptotic vesicles reconstituted β_2m-deficient APCs for MHC-I-restricted antigen presentation. Moreover, we

demonstrated activation of antigen-specific CD4 T cells by apoptotic vesicles. MHC-II-restricted CD4 T cells, which are activated by exogenous antigens through the endosomal pathway, play an important role in protective immunity against tuberculosis. Crosspriming is also crucial for CD4 T cell activation in tuberculosis, since infected macrophages are hampered in MHC-II presentation due to mycobacterial interference with IFN-γ signaling (Wojciechowski et al., 1999).

Recent data suggest that DCs are responsible for crosspriming of CD8 T cells in vivo (den Haan et al., 2000; den Haan and Bevan, 2002). Since we observed T cell activation in draining lymph nodes, we experimentally determined how the antigen was transported from the periphery to the lymphatic tissue. After subcutaneous injection of fluorescent vesicles, subsequent staining of DCs and macrophages in draining lymph node sections demonstrated exclusive allocation of vesicles to DCs. Uptake of particulate antigen by DCs in peripheral tissue for subsequent transport to draining lymph nodes is in accordance with studies showing that soluble rather than particulate antigens can be directly drained by the lymph for cell-independent transport to secondary lymphoid organs (Itano and Jenkins, 2003). Moreover, immunization of CCR7-deficient animals with apoptotic vesicles failed to induce CD8 T cell activation. CCR7 is expressed on naive and memory T cells as well as activated DCs and is critically required for homing to lymphatic tissue (Martin-Fontecha et al., 2003). Main ligands for CCR7 are CCL21 and CCL19, which are produced by endothelial cells of lymphatic vessels and high endothelial venules as well as stromal cells and mature DCs of lymphatic T cell zones. Our results demonstrate that homing of DCs is a prerequisite for crosspriming of CD8 T cells by apoptotic vesicles.

Processing of antigen contained in apoptotic vesicles in recipient DCs mainly involved endosomal mechanisms rather than proteasomal cleavage as shown by chemical inhibition of either pathways. This contrasts recent data that found dependency on TAP and proteasomes of crosspriming by cell-associated antigens (Fonteneau et al., 2003). Two options of antigen processing independently from proteasomes can be envisaged. First, OVA could be present in apoptotic vesicles in an already processed form directly available for MHC-I binding. We consider this option unlikely because we identified complete OVA molecules by immunoblot analysis of apoptotic vesicles. More importantly, recent studies revealed the nature of crosspriming antigens defined by maturity of proteins (Shen and Rock, 2004). Unstable peptides with a shorter half-life than mature proteins were insufficient for crosspresentation (Wolkers et al., 2004; Norbury et al., 2004). Second, OVA could be processed in recipient APCs independent from proteasomal cleavage. Indeed, recent experiments identified an exclusive endosomal mechanism of antigen editing for crosspresentation mediated by cathepsin S not involving TAP and proteasomes (Shen et al., 2004; Rock and Shen, 2005).

Apoptotic vesicles represent membrane structures, which supposedly are disintegrated as prerequisite for further processing of their antigenic content. Saposins A-D are lipid transfer proteins ubiquitiously present in lysosomes generated by cleavage from the common precursor prosaposin (Schuette et al., 2001). Saposins exhibit a dual function in binding to proteins as well as lipids, and they are essential cofactors of enzymes involved in lipid degradation (O'Brien and Kishimoto, 1991; Kolter and Sandhoff, 2005). Moreover, saposins bind to phospholipid membranes, thereby destabilizing the integrity of intralysosomal vesicles, which renders vesicular constituents accessible for further processing (Vaccaro et al., 1995). Previous work described that saposins are capable of extracting antigens from membranes (Winau et al., 2004b). Due to the structural analogy between intralysosomal and apoptotic vesicles and the high affinity of saposins to phosphatidylserine exposed on apoptotic membranes (Henson et al., 2001), we analyzed prosaposin-deficient APCs for antigen presentation of apoptotic vesicles. Indeed, prosaposin-deficient DCs failed to activate CD8 T cells by vesicles from mycobacteria-infected macrophages compared to wild-type APCs. Reconstitution of saposin-deficient APCs by exogenous prosaposin restored their antigen-presenting capacity. Thus, saposins perform vital functions in disintegration of apoptotic vesicles for crosspriming of CD8 T cells.

Activation and maturation of APCs is an essential precondition for efficient T cell activation. In infectious disease, TLRs on DCs and macrophages mediate APC activation through binding of pathogen components (Medzhitov et al., 1997). TLRs are pattern recognition receptors (PRR) that sense the presence of pathogens in the macroorganism by binding of conserved pathogenic structures, termed pathogen-associated molecular patterns (PAMP), such as LPS and BLP (Medzhitov, 2001). We observed potent adjuvanticity of apoptotic vesicles from BCG-infected macrophages by activation through TLR-2 and, in DCs, TLR-4. Immunoblotting of apoptotic vesicle proteins revealed candidate molecules for the adjuvant effect such as 19 kDa lipoprotein known to stimulate through TLR-2 (Ciaramella et al., 2004; Lopez et al., 2003). Because apoptosis permanently takes place in the macroorganism to ensure homeostasis of tissues and organs (Hengartner, 2000), cell death is physiologically not associated with signals that entail immune responses. Thus, the silencing function of apoptosis is essential to preclude inflammation and autoimmune disease. For example, necrotic instead of apoptotic tumor cells induce crosspriming of CD8 T cells (Sauter et al., 2000). However, in infectious disease, apoptotic vesicles are equipped with a multitude of PAMPs. Thus, pathogen-derived adjuvant molecules override the silencing mechanisms of apoptosis by activation of APCs through TLRs, subsequently leading to protective immune responses.

Tuberculosis is still a major global health threat, claiming 2 million deaths per year (Kaufmann and McMichael, 2005). Although efficient antibiotics are available, the complex regimen requires high compliance of patients over several months and, thus, treatment is frequently unsuccessful. Moreover, increased incidences of multidrug-resistant strains complicate therapy. Therefore, development of a novel efficacious vaccine is crucial to control tuberculosis, taking into account that the standard vaccination with the attenuated strain *M. bovis* BCG only prevents severe forms of childhood disease (Kaufmann, 2001). Our results demonstrate that vaccination

with apoptotic vesicles from mycobacteria-infected macrophages induces protection against *M. tuberculosis* compared to treatment with naive vesicles. In a combinatory approach linking vaccination and comparative proteome analysis, novel antigen candidates can be deduced from the mycobacterial proteome of apoptotic vesicles for future vaccination strategies.

Taken together, apoptotic vesicles represent an autonomous immunological entity since they carry the triad of antigen, antigen-presenting molecule, and adjuvant for effective T cell activation. We propose the detour pathway to reflect a genuine immunological mechanism for crosspriming of T cells in vivo and protection against tuberculosis.

Experimental Procedures

Experimental Animals and Bacteria

6- to 8-week-old C57BL/6, OT-1, OT-2, β_2m-, TLR-2-, TLR-4-, and MyD88-KO mice were bred under specific pathogen-free conditions in our facility at the Federal Institute for Health Protection of Consumers and Veterinary Medicine (Berlin, Germany). OT-1 and OT-2 breeder mice were obtained from The Jackson Laboratories (Bar Harbor, ME). OT-1 mice express a transgenic TCR, which recognizes OVA residues 257–264 in the context of H-2K^b (Hogquist et al., 1994). TCR-transgenic OT-2 mice are specific for the I-A^b restricted peptide 323–339 of OVA (Barnden et al., 1998). CCR7-knockout mice (Forster et al., 1999) were a kind gift from Dr. M. Lipp (Max-Delbrueck-Centre for Molecular Medicine, Berlin, Germany), and pSAP-deficient animals were bred at the Kekulé Institute (Bonn, Germany). *M. tuberculosis* H37Rv, *M. bovis* BCG (Danish strain), and recombinant *M. bovis* BCG-OVA were grown in 7H9 medium complemented with albumin, dextrose, and catalase (ADC, Difco, Germany). BCG-OVA was recombinantly engineered with a partial sequence of the OVA gene (codons 230–359) under the control of the HSP60 promoter (Dudani et al., 2002).

Infection, Apoptotic Vesicle Preparation, and Labeling

BCG or BCG-OVA were used for infection of macrophages at midlog phase of bacterial growth (A_{600} = 0.7) at an moi of 10:1. Bacterial lysates were generated by sonication for 10 min at full level at 4°C with a Branson sonifier (Danbury, CT) equipped with a Titanium sonication cup. Infection was performed for 4 hr in the presence of 5% horse serum. Apoptotic vesicles were generated from either noninfected or BCG-infected macrophages cultured for 4–6 days in the absence of FCS. Deprivation of FCS induced apoptotic vesicles in both groups of macrophages. We described lower apoptosis of macrophages after infection with BCG as compared to *M. tuberculosis* (Schaible et al., 2003). Yet, by extending the culture period of infected cells, BCG sufficiently induced apoptotic cell death. Purification of vesicles was realized by consecutive centrifugations at 800 × g, 1,800 × g, 25,000 × g, and 100,000 × g for 1 hr. High-speed pellets were free of mycobacteria as verified by culture. From 4×10^8 macrophages, we purified vesicles equal to either 2.5–2.8 mg from noninfected or 4.1–5 mg from infected cultures. All antigen preparations were adjusted to equivalent protein content of either 50 μg/ml for immunization or 5 mg/ml for in vitro experiments as indicated (BCA assay, Pierce, USA). Apoptotic vesicles were coupled to Texas red (Molecular Probes, Netherlands) by hydrazide labeling according to the manufacturer's protocol.

Fluorescence, Confocal, and Electron Microscopy

Macrophages (1×10^5 per well) were seeded on 8-well multichamber slides (Nalge Nunc International, USA). Subsequently, cells were infected, stained for Annexin V, fixed with 4% paraformaldehyde (PFA), and embedded in Mowiol for analysis by an DM-IRBE fluorescence microscope (Leica, Germany). For confocal microscopy, lymph node sections were mounted on slides, stained with anti-CD11c-Alexa-488 (N 418) and anti-F4/80 coupled to Cy5, embedded in Mowiol, and examined by a laser scanning microscope (Leica TCS-NT, Germany). For scanning electron microscopy, macrophages or apoptotic vesicles were fixed with 2.5% glutaraldehyde and postfixed by repeated incubations with 1% osmium tetroxid/1% tannic acid. After dehydration with a graded ethanol series, specimens were critical-point dried and coated with 2 nm platinum before analysis on a Leo 1550 scanning electron microscope.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/24/1/105/DC1/.

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